

**PHYTOCHEMICAL, ANTIOXIDANT AND CYTOTOXICITY OF
HYDROETHANOLIC EXTRACTS OF *CROTALARIA RETUSA* L.**

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ABSTRACT

For centuries now, plants have served as a great source of compounds with pharmacological properties. Some plants like weeds, however, have not been fully exploited for their medicinal value. In this research, the phytochemical, total phenolic content, antioxidant and antiproliferative effect of *Crotalaria retusa*, a weed reported to have potential to fight tumours was assessed. The study focused on the leaf, stem, seed, pod and flower of this plant as well as fractions of its stem was assessed. Standard methods such as the MTT, the DPPH and the Folin-Ciocalteu assays were used. The phytochemicals present included saponins, tannins, alkaloids and sterols. The leaf was found to have the highest concentration of phenols (67.35 ± 1.153 mg GAE/g of extract) and the best free radical scavenging activity with an EC_{50} value of 0.222 ± 0.004 mg/mL. All the extracts induced cytotoxicity in a

dose dependent manner. The study revealed that the stem of *C. retusa* exhibited the highest cytotoxicity against the selected cancer cells. However, extracts of the plant parts were observed to be non-selective towards cancer cells since they were equally toxic to the normal human liver (WRL 68) cells. All the fractions tested against the cancer cells did not exhibit any significant increase in cytotoxicity. The study reveals the antiproliferative nature of *C. retusa*, with Jurkat being the most sensitive cell line. The antioxidant potential of this plant could be due in part, to its total phenol content. Further study is however needed to determine the active principle of *C. retusa*.

KEYWORDS: *Crotalaria retusa*, Phytochemical, Antioxidant Cytotoxicity, Jurkat.

INTRODUCTION

Plants are nature's gift to mankind in terms of providing us with food, oxygen, as well as shelter. Since time immemorial, they have served as the first line of defence used by our forefathers to fight diseases. Most orthodox drugs administered today were derived from plants (Ncube *et al.*, 2008).

The plant *Euphorbia peplus* contains a compound called ingenol mebutate (Picato) which is used to treat skin cancer (Zarchi and Jemec, 2015). The common drug quinine was also derived from the bark of *Cinchona officinalis*, and this drug is widely prescribed for the treatment of malaria in countries that cannot afford the more expensive anti-malaria drugs (Reyburn *et al.*, 2009). Some types of plants have been under-utilized and are as such destroyed when they appear above the ground. Typical examples are weeds and ornamentals. Native to Africa, the weed *Crotalaria retusa* L., commonly called the devil bean or rattle box, is one of the numerous weeds in the Fabaceae family. It is a legume that has the capability of accumulating monocrotaline, an important toxicant with a wide degree of toxicity in animals (World Health Organization, 1988). This compound has been suggested to have a potential for killing tumours since it is capable of killing lung cells (Schoental and Head, 1955). Very little is however known of its antiproliferative activity on cancerous cells.

Cancer incidence and mortality has quickly increased over the past decade and is gradually becoming a menace especially in less developed countries. Most patients in less developed countries are unable to treat the disease mostly due to the high cost involved. Assessing the anticancer potential of these locally available plants brings us one step closer to discovering an alternative source of chemotherapy against cancer. This will possibly be less expensive and affordable since these plants are readily available for preparation and administration.

The objective of this research was to assess the antiproliferative activity of 50% hydroethanolic extracts of *C. retusa* parts. The phytochemical, antioxidant and total phenolic content (TPC) of its leaf, stem, pod, seed and flower were analyzed.

MATERIALS AND METHODS

Cell lines and reagents

The cell lines used (Jurkat, MCF 7, PC 3, WRL 68, HepG2) were obtained from RIKEN BioResource Centre Cell Bank (Japan). Culture media (RPMI and α -MEM), 96 well plates, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, isopropanol, HCl, trypan blue solution, absolute ethanol, foetal bovine serum (FBS), antibiotics (penicillin and streptomycin), 2, 2-diphenyl-1-picryl hydrazyl (DPPH) and phosphate buffer saline were obtained from Sigma-Aldrich Company (St. Louis, MO, USA).

Plant Preparation

C. retusa samples were handpicked from the Tech Credit Union Building environs (6°40'13.7"N 1°34'28.2"W), on the KNUST campus in April, 2014 before 9.00 am. Specimen of the plant was sent to the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi for authentication by a taxonomist and a voucher specimen was deposited at the Herbarium for reference purpose (KNUST/HMI/2014/L092). The plant was sorted into leaf, pod, seed, flower and stem. The stem component was chopped into pieces and all the parts were washed separately with water three times and air dried at room temperature for three weeks. The dried samples were separately pulverized and packaged in zip-locks for further use. Preparation of 50% hydroethanolic extracts of the various parts was carried out separately, by suspending 50 g of the powder of each part in 500 mL of 50% ethanol (50:50 v/v). The extraction was done by cold maceration for 24 hrs at room temperature on a shaker. The extracts were filtered through cotton wool, concentrated using a rotary evaporator and freeze-dried to obtain the *C. retusa* hydroethanolic leaf, pod, seed, flower and stem crude extracts.

Fractionation of hydroethanolic stem extract

Fractionation of the hydroethanolic stem extract of *C. retusa* was carried out in a separating funnel using solvents of increasing polarity: petroleum ether, chloroform and ethyl acetate. A mass of 2.5 g of crude hydroethanolic stem extract of *C. retusa* was dissolved in 25 mL of 50% ethanolic solution and was successively partitioned with petroleum ether, then with chloroform and finally with ethyl acetate, each having a volume of 50 mL, to obtain petroleum ether, chloroform and ethyl acetate fractions. This was done for two to three times as polarity increased. The remaining portion was designated as hydroethanolic fraction.

Phytochemical Screening

The presence of general glycosides, anthracene glycosides, saponins, tannins, alkaloids, flavonoids, sterols and triterpenoids was analyzed using standard methods (Trease and Evans, 1989; Sofowora, 1993; Harborne, 1998).

Determination of total phenols

Total phenolic content (TPC) was determined using the Folin–Ciocalteu assay with slight modification (Marinova *et al.*, 2005). To a volume of 10 µL of sample, 790 µL of distilled water was added. The concentration of the crude extracts tested was 5 mg/mL. A volume of 50 µL of Folin–Ciocalteu reagent was added to the diluted samples and thoroughly mixed. The mixtures were incubated in the dark for 8 mins. Subsequently, 150 µL of 7% Na₂CO₃ was added before incubation of the mixture for 2 hrs in the dark at room temperature. Triplicate experiments were performed. The absorbance was read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). Gallic acid (GA) was used as the standard phenolic compound. A GA calibration curve was plotted and used to determine the total phenolic content. The results were expressed in milligrams of GA equivalents per gram dry mass (mg GAE/g DM).

Antioxidant assay

The antioxidant activity of *C. retusa* leaf, pod, seed, flower and stem extracts was determined using the free radical scavenging activity by DPPH method with some modification (Blois, 1958). Methanolic solution of DPPH (0.5 mM) was added to equal volumes of various concentrations of each extract (concentration range 0-5 mg/mL). After 20 mins incubation at room temperature, the absorbance was read at a wavelength of 517 nm (Tecan Infinite M200 Pro plate reader, Austria). The inhibition concentration at 50% (IC₅₀) value of each extract was calculated from the following formula.

$$\% \text{ Antioxidant activity} = [(A_0 - A_1)/A_0 \times 100]$$

where A₀ is the absorbance of negative control (methanol) and A₁ is the absorbance of test sample with DPPH. Butylated hydroxytoluene (BHT) was used as standard control. Triplicate experiments were performed. The EC₅₀ value, which is the concentration of the extracts that can cause 50% free radical scavenging activity, was determined.

MTT assay

L-RPMI and α -MEM culture media respectively, supplemented with 10% foetal bovine serum (FBS) containing penicillin, streptomycin and L-glutamine were maintained in culture at 37°C in a humidified 5% CO₂ atmosphere. The tetrazolium-based colorimetric assay (MTT) was used to determine the cytotoxicity of *C. retusa* on the cancer and normal cell lines (Ayisi *et al.*, 2011). Cells were seeded into the 96-well plates at the concentration of 1×10^4 cells/well, treated with varying concentrations of the plant extracts (0-1000 μ g/mL) and incubated as indicated above for 72 hrs. A color control plate was also setup for each extract including the positive control, curcumin. MTT solution (0.5 mg/mL) was added to each well on the plate, and incubation continued for further 4 hrs. The reaction was stopped with acidified isopropanol solution, and the plate incubated in the darkness overnight at room temperature before reading the absorbance at 570 nm using a microplate reader (Tecan Infinite M200 Pro, Austria). The percentage cell viability was determined as follows.

$$\% \text{cell viability} = \text{mean absorbance} \left(\frac{\text{treated cells} - \text{blank}}{\text{untreated cells} - \text{blank}} \right) \times 100$$

The IC₅₀ values were determined from the plot of percent cell viability on the y-axis against extract concentrations on the x-axis.

Statistical analysis

Data were analyzed by one-way analysis of variance and the means assessed by Tukey's test at 5% level of significance ($p < 0.05$) using Graph pad Prism version 5.0.

RESULTS**Phytochemical screening**

Table 1 shows the presence and levels (marked by intensity of colour or froth) of general glycosides, anthracene glycosides, saponins, tannins, alkaloids, flavonoids, sterols and triterpenoids. In all the samples tested for, only the stem showed the presence of general glycosides. None of the samples, however was observed to possess anthracene glycosides.

Table 1: Phytochemical constituents present in 50% hydroethanolic crude extracts of *C. retusa* parts.

Phytochemical	Seed	Pod	Flower	Stem	Leaf
General glycoside	++	++	+++	++	++
Anthracene glycoside	-	-	-	-	-
Saponins	+++	+++	+++	+++	+++
Tannins	+++	+++	+++	+++	+++
Alkaloids	+++	++	++	++	++
Flavonoids	+++	+++	+++	+++	-
Sterols	+	+++	+++	++	++
Triterpenoids	-	+++	-	-	-

The data show the intensities of observed colours or froths as compared to standard. +++ present at high concentration; ++ present in moderate concentration; + present in low concentration; - absent.

Total Phenol Content

From the standard linear plot constructed, the total phenolic content of all the plant extracts were extrapolated. The leaf of the *C. retusa* plant recorded the highest concentration of phenols (67.35 ± 1.153 mg Gallic acid equivalent/g) while the seed was least in total phenolic content. The total phenol content of the leaf, stem, seed, pod and flower is shown in Table 2.

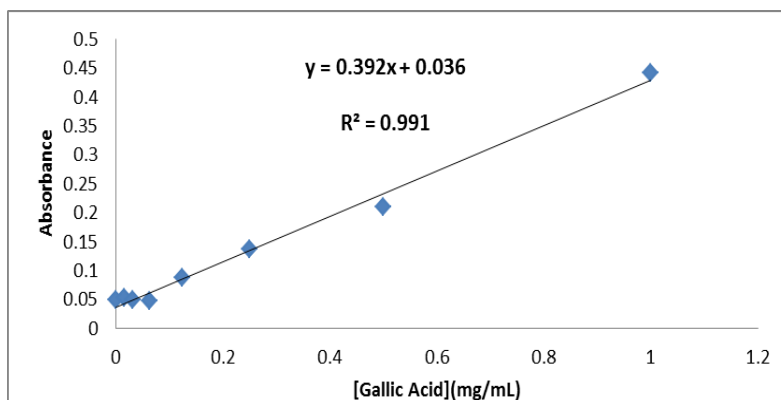


Figure 1: Standard calibration plot obtained from the various gallic acid concentrations.

Table 2: Total phenolic content of hydroethanolic extracts of *C. retusa*.

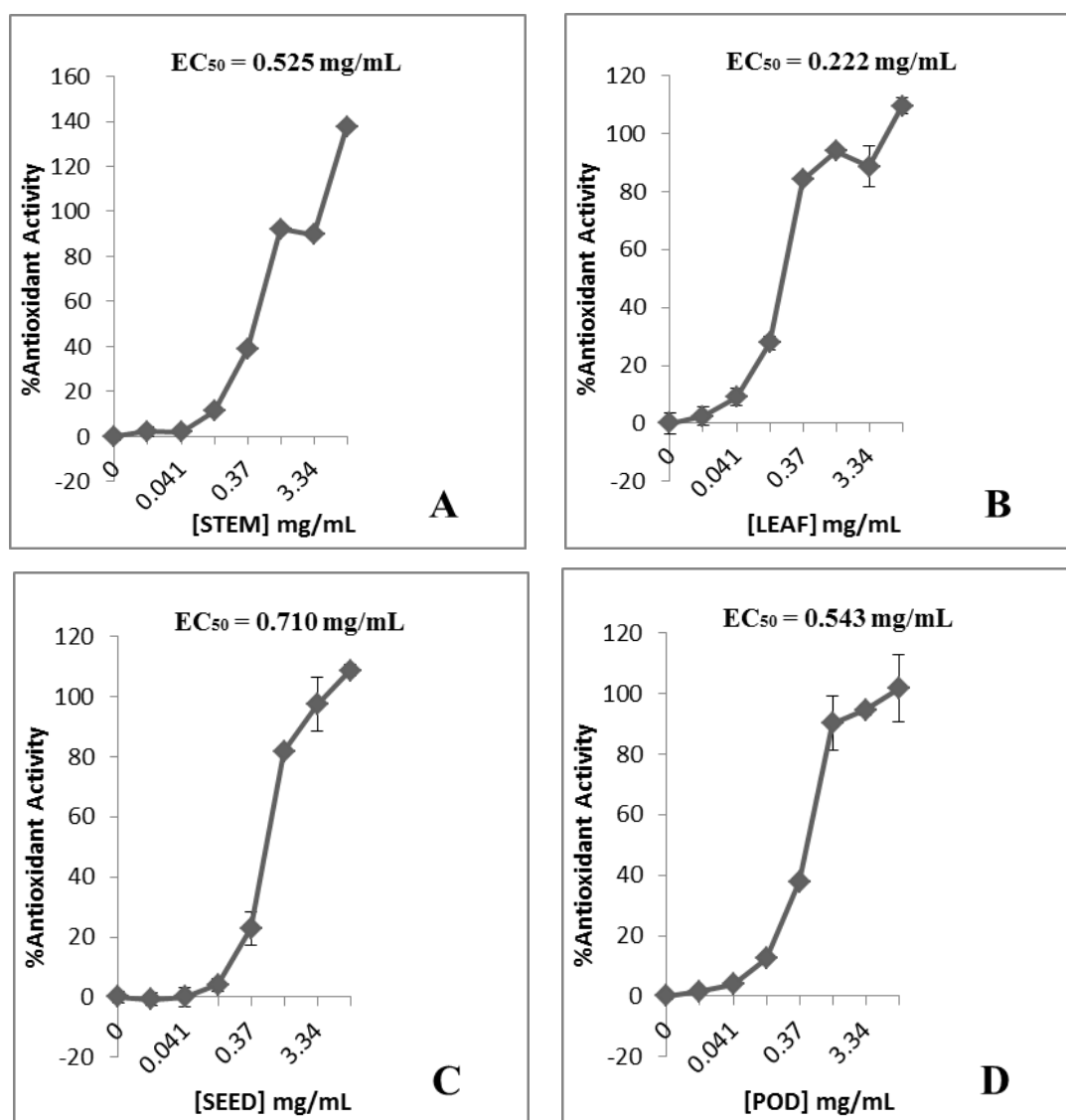
Plant	Part	Total phenolic content (mg GAE/g)	P-value
<i>C. retusa</i>	Seed	34.70 ± 0.573	< 0.0001
	Pod	61.22 ± 0.0	
	Flower	37.15 ± 0.870	
	Stem	50.62 ± 0.290	
	Leaf	67.35 ± 1.153	

Antioxidant activity

The ability of the extracts to scavenge DPPH free radical was used to assess its antioxidant activity. The leaf of *C. retusa* exhibited the strongest antioxidant activity with an EC_{50} value of 0.222 mg/mL. The antioxidant activity of the standard (BHT), stem, leaf, flower, seed and pod of *C. retusa* is shown in figure 2.

Antiproliferative activity of extracts and curcumin

All the extracts exhibited cytotoxicity towards Jurkat, MCF 7, PC 3 and WRL 68 in a dose dependent manner. Only the stem extract was active against all the tested cell lines with its strongest inhibition against Jurkat (IC_{50} value = 221.97 μ g/mL). Curcumin inhibited the growth of the normal cell (WRL 68), likewise the pod, flower, stem and leaf. Figures 3, 4, 5 and 6 show the antiproliferative activity of the parts of *C. retusa* on the cells.



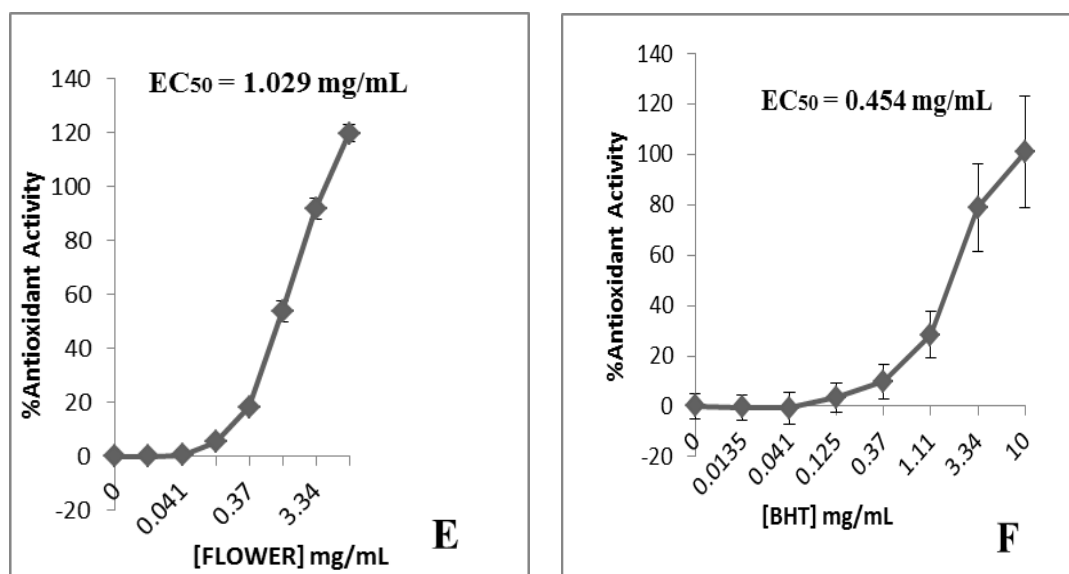
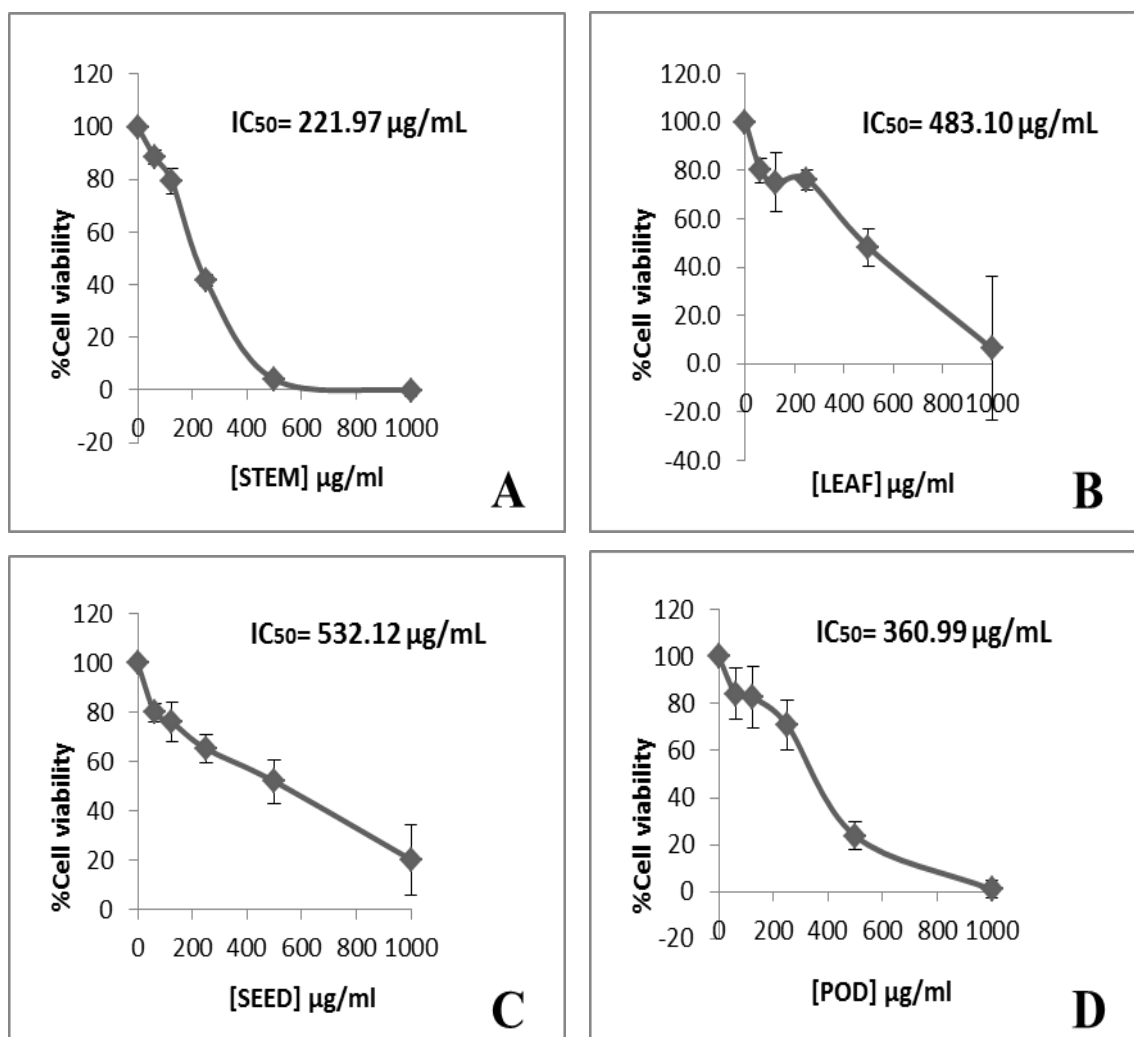


Figure 2: Antioxidant activity of (A) *C. retusa* stem, (B) *C. retusa* leaf, (C) *C. retusa* seed, (D) *C. retusa* pod, (E) *C. retusa* flower and (F) BHT. Each point represents a mean of three determinations.



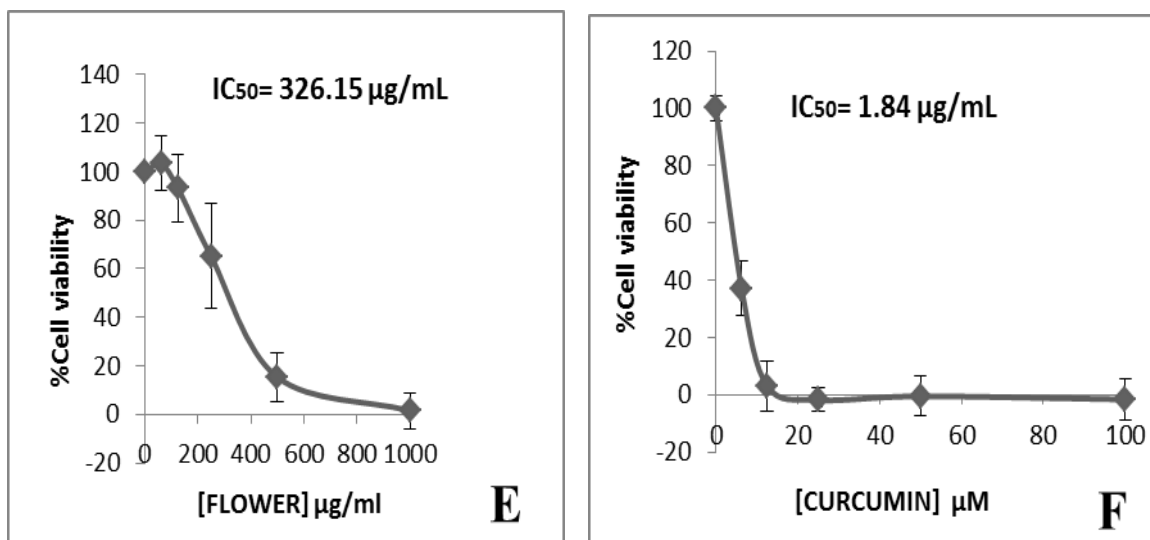
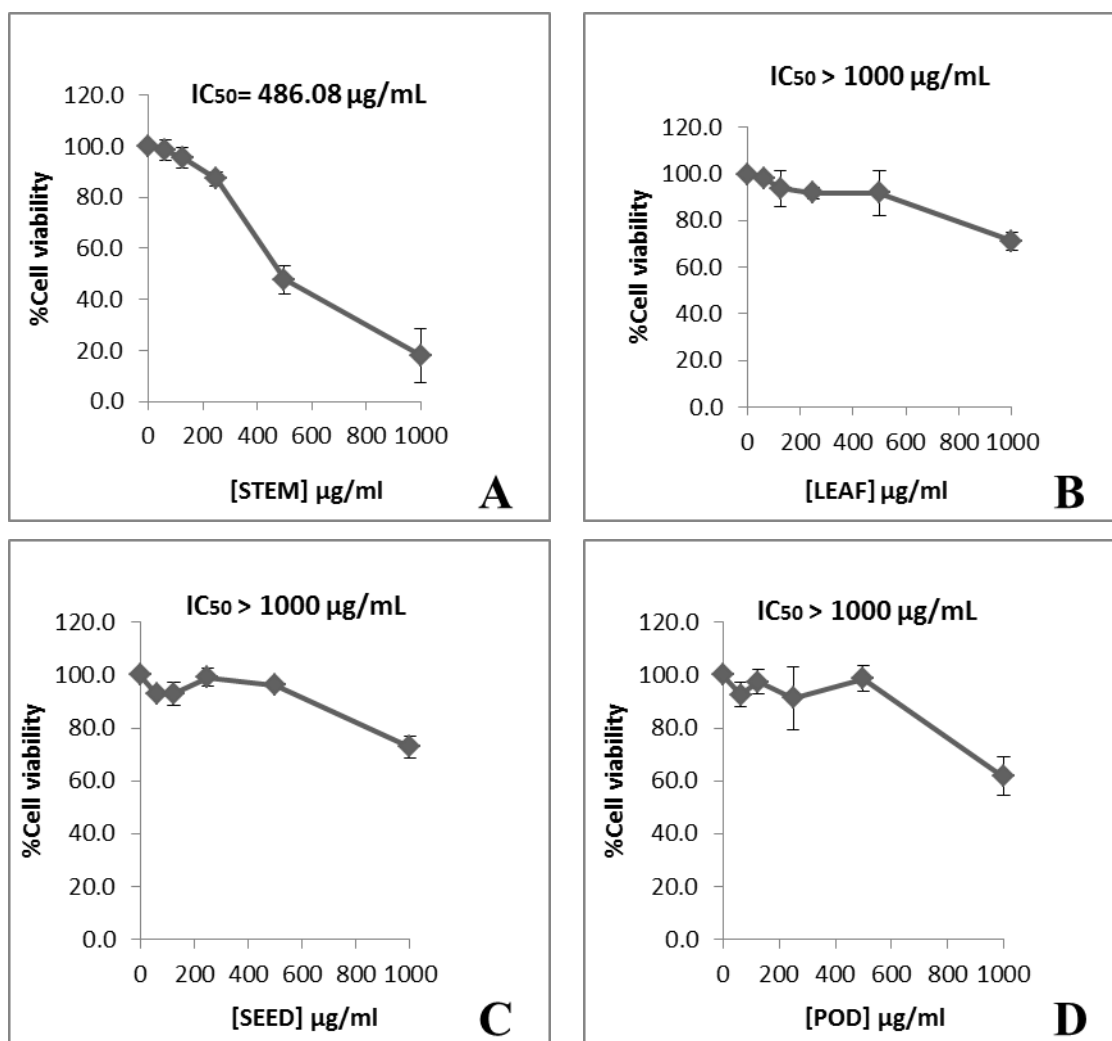


Figure 3: Antiproliferative activity of (A) *C. retusa* stem, (B) *C. retusa* leaf, (C) *C. retusa* seed, (D) *C. retusa* pod, (E) *C. retusa* flower and (F) Curcumin on Jurkat cells. Each point represents a mean of three determinations.



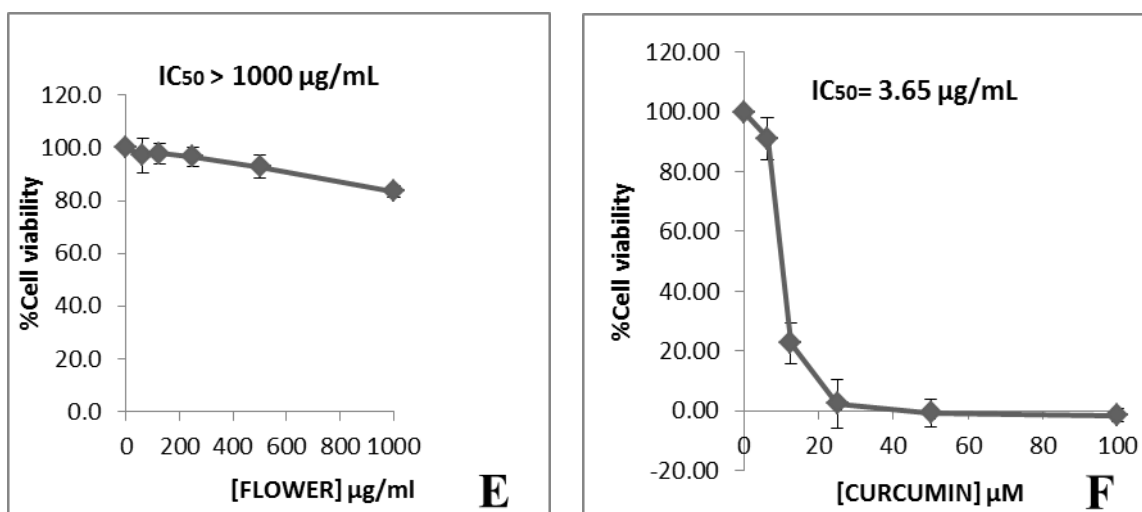
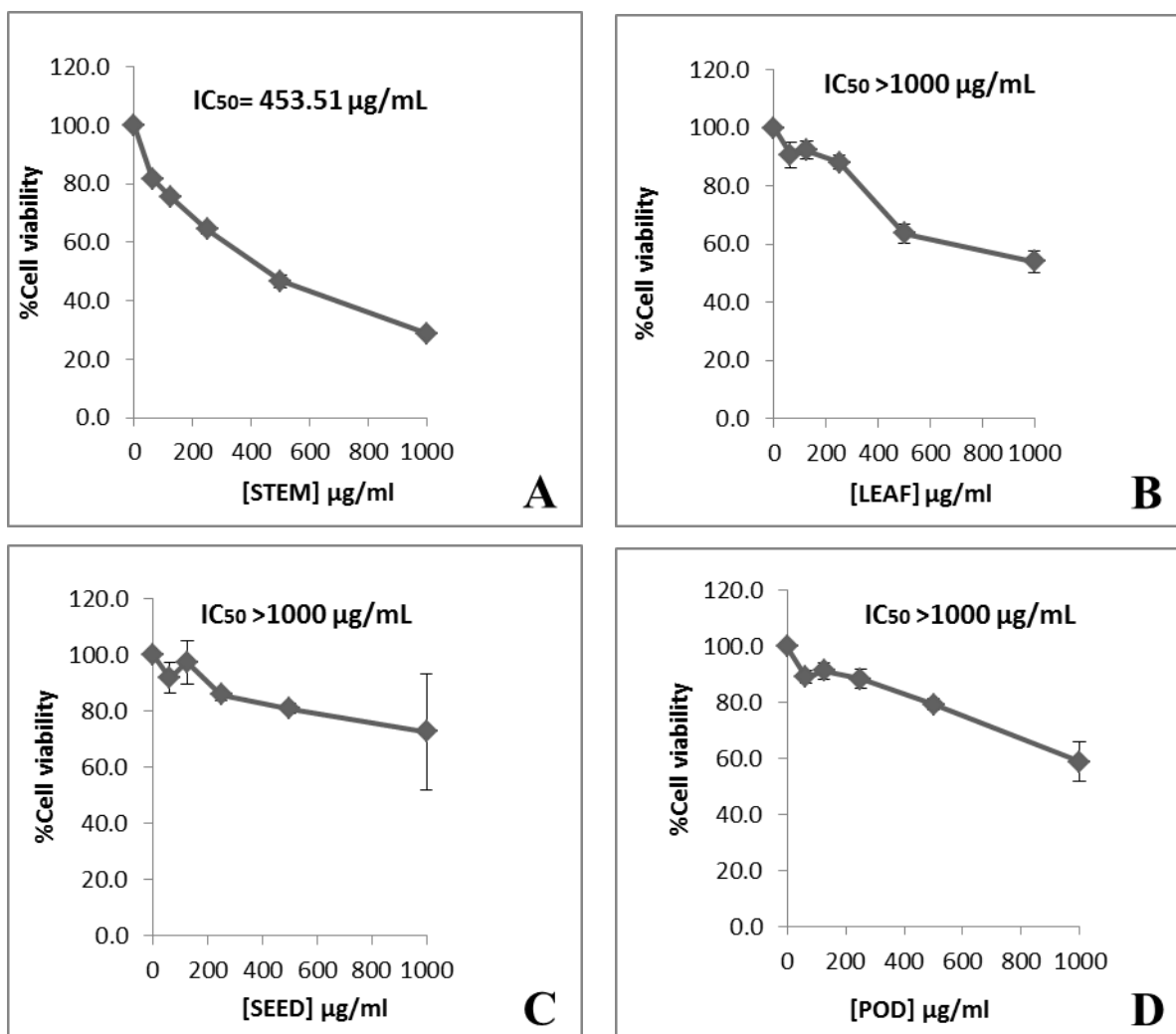


Figure 4: Antiproliferative activity of (A) *C. retusa* stem, (B) *C. retusa* leaf, (C) *C. retusa* seed, (D) *C. retusa* pod, (E) *C. retusa* flower and (F) Curcumin on MCF 7 cells. Each point represents a mean of three determinations.



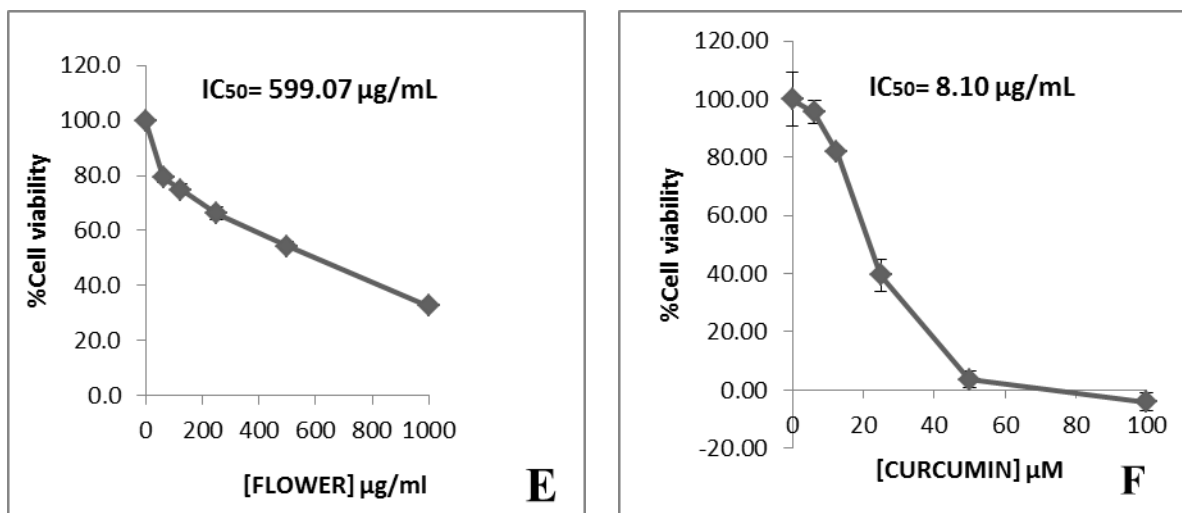
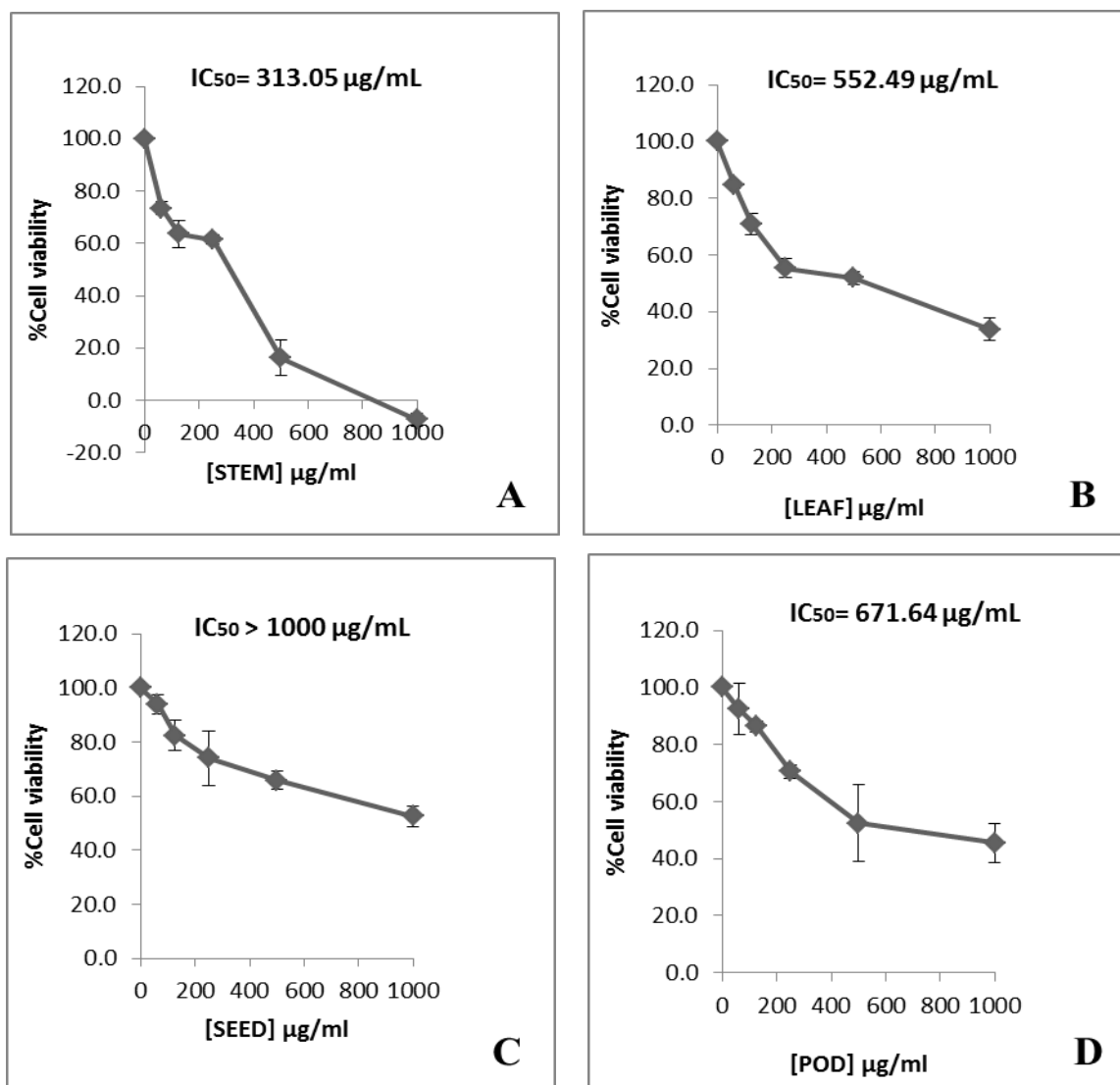


Figure 5: Antiproliferative activity of (A) *C. retusa* stem, (B) *C. retusa* leaf, (C) *C. retusa* seed, (D) *C. retusa* pod, (E) *C. retusa* flower and (F) Curcumin on PC 3 cells. Each point represents a mean of three determinations.



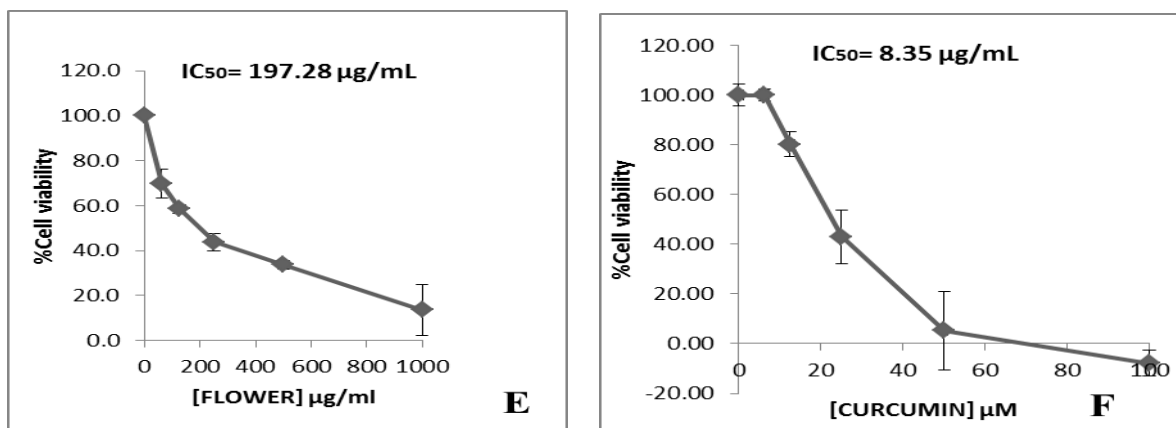
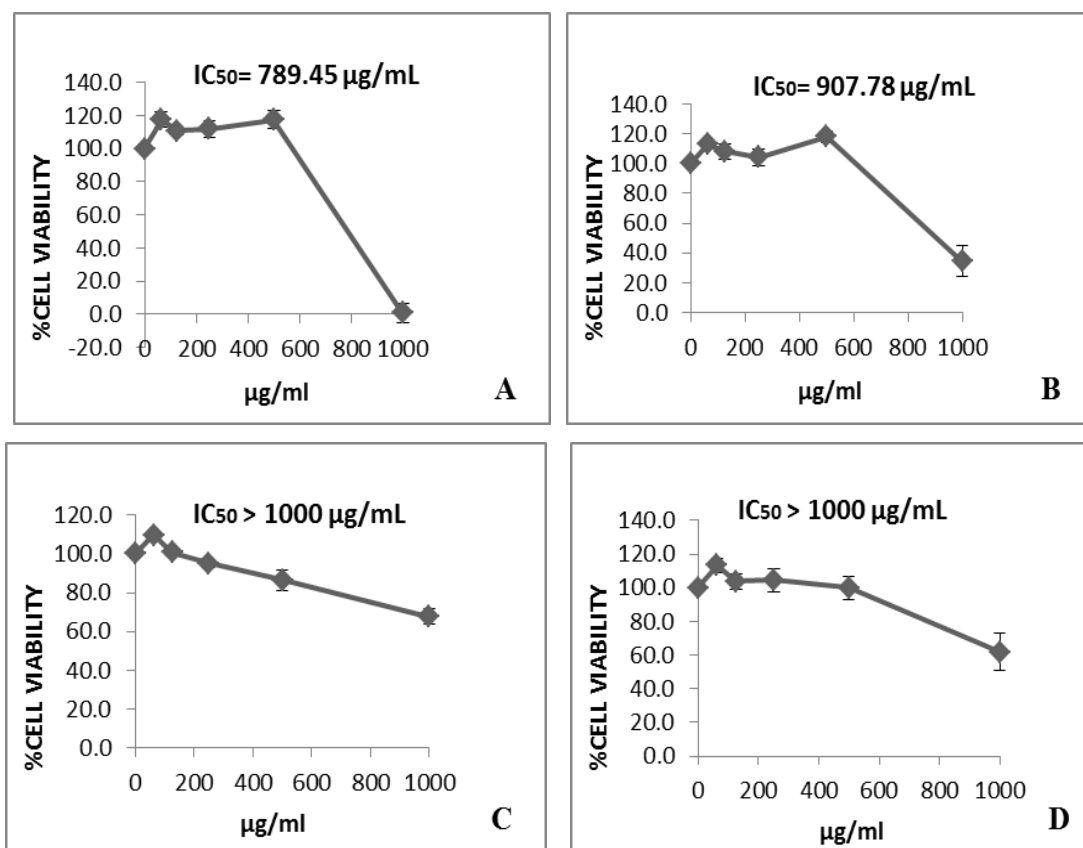


Figure 6: Antiproliferative activity of (A) *C. retusa* stem, (B) *C. retusa* leaf, (C) *C. retusa* seed, (D) *C. retusa* pod, (E) *C. retusa* flower and (F) Curcumin on WRL 68 cells. Each point represents a mean of three determinations.

Antiproliferative activity of *C. retusa* stem fractions and curcumin

Petroleum ether, chloroform, ethyl acetate and hydroethanolic fractions of the most active part of the plant, its stem, were also assessed for their level of cytotoxicity. Figures 7, 8 and 9 show the antiproliferative activity of these fractions on Jurkat, MCF 7 and HepG2 cells respectively.



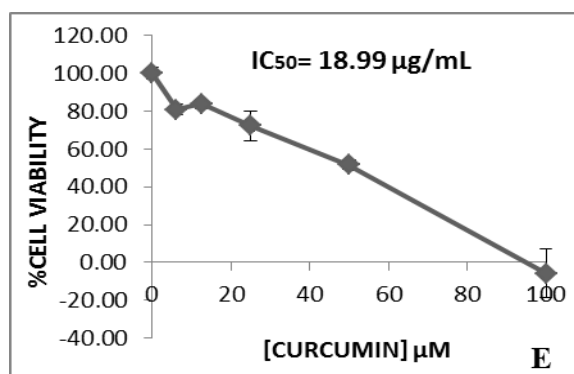


Figure 7: Antiproliferative activity of *C. retusa* stem petroleum ether (A), chloroform (B), ethyl acetate (C), hydroethanolic (D) fractions and curcumin (E) on Jurkat cells. Each point represents a mean of three determinations.

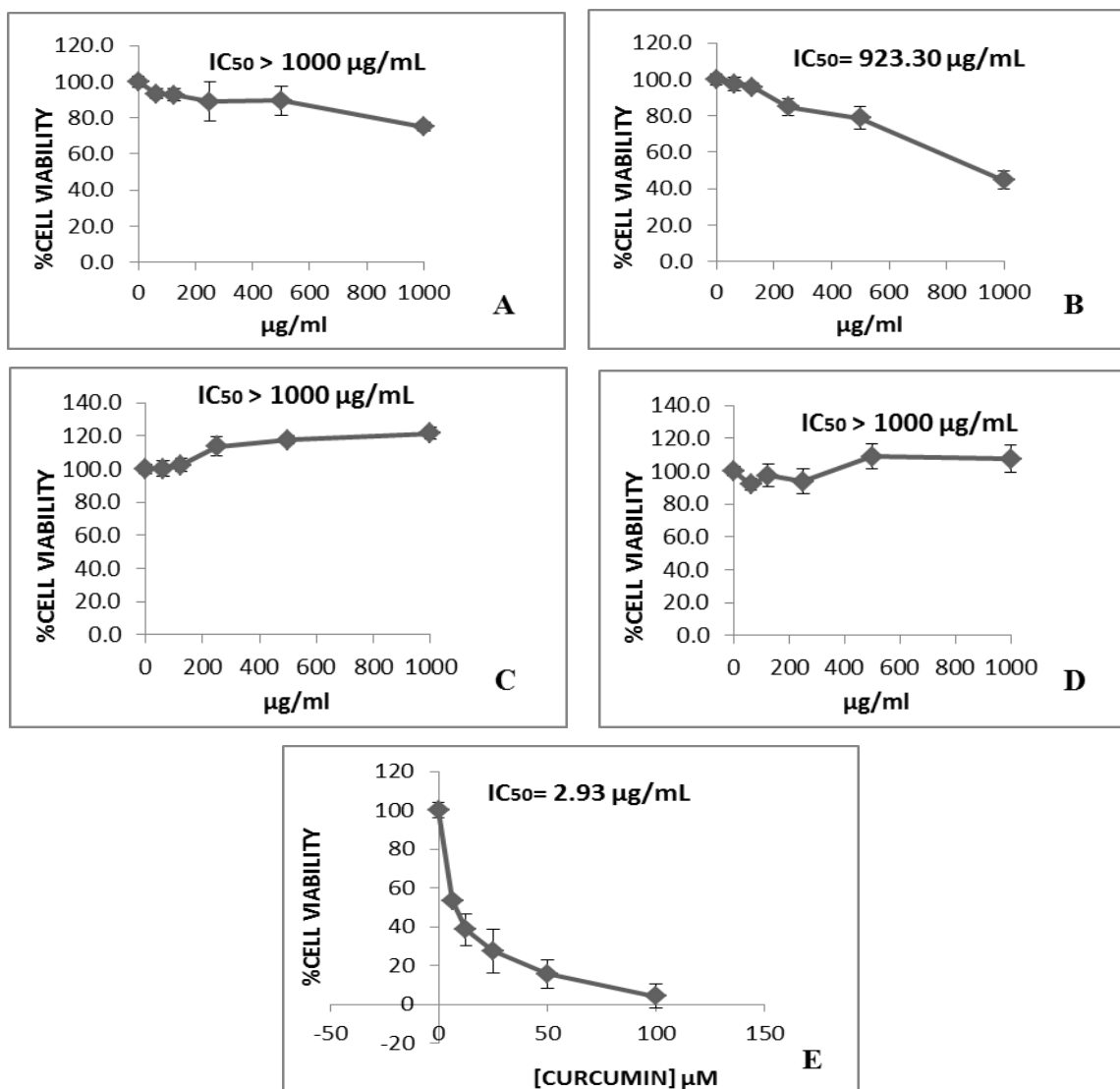


Figure 8: Antiproliferative activity of *C. retusa* stem petroleum ether (A), chloroform (B), ethyl acetate (C), hydroethanolic (D) fractions and curcumin (E) on MCF 7 cells. Each point represents a mean of three determinations.

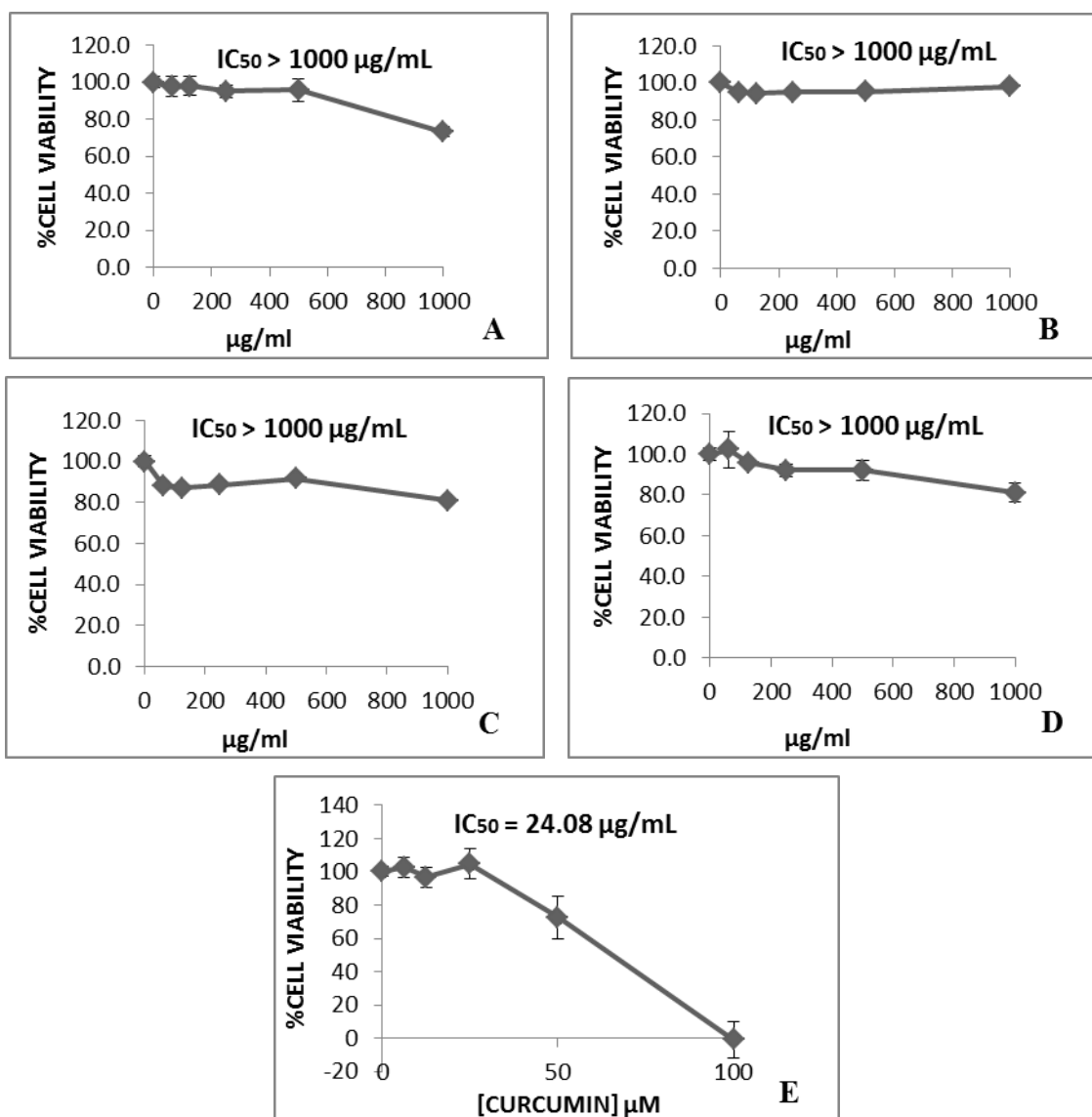


Figure 9: Antiproliferative activity of *C. retusa* stem petroleum ether (A), chloroform (B), ethyl acetate (C), hydroethanolic (D) fractions and curcumin (E) on HepG2 cells. Each point represents a mean of three determinations.

DISCUSSION

All the crude extracts of the various parts of the plant considered expressed a rather high level of saponins and tannins, with alkaloids and sterols varying in concentration. Earlier studies have shown the presence of alkaloids and sterols in the leaf of *C. retusa* (Dhole *et al.*, 2012). The presence of alkaloids and sterols in the leaf of this plant was confirmed in this research. Flavonoids and triterpenoids were absent from the leaves of this plant. Only the stem of *C. retusa* was observed to contain general glycosides.

Phytochemicals such as phenols and polyphenolic compounds like flavonoids are generally present in medicinal plants and these compounds have been shown to possess good antioxidant activities (Van Acker *et al.*, 1996). An earlier study revealed that *C. retusa* possessed the strongest antioxidant activity compared with other *Crotalaria* species (Devendra *et al.*, 2012). Results from this research showed that *C. retusa* indeed has great antioxidant activity, predominantly found in its leaf. There was significant difference between the observed total phenolic contents of the various parts analyzed (p-value < 0.0001).

Devendra *et al.* (2012) indicated compounds isolated from ethanolic extracts of *Crotalaria* species, possess pharmacological properties and potential to develop natural compound-based pharmaceutical products. The findings of this study confirm the presence of potential compounds with the ability to scavenge free radical and potential anti-carcinogenic and anti-inflammatory agents because of the observed high antioxidant activity (Stavric, 1993; Elangovan *et al.*, 1994; Marrin *et al.*, 2002).

The MTT cell viability assay showed that only the stem of this plant had antiproliferative activity against all the three cancer cells (Jurkat, MCF 7 and PC 3). Jurkat was most sensitive to all the *C. retusa* extracts used in this research compared to the other cells.

The seed of *C. retusa* has been reported by Maia *et al.* (2013), to have high levels of the hepatotoxic alkaloid, monocrotaline, which requires bioactivation to become toxic to hepatocytes (John *et al.*, 2005). Thus, the flower (other than the seed) of *C. retusa* was the most hepatotoxic part of this plant (toxic to WRL 68 cells), even though the seed has the highest levels of monocrotaline. These findings suggest that indeed, monocrotaline, though hepatotoxic would require bioactivation (John *et al.*, 2005), hence its reduced toxicity *in vitro*. The flower could possibly contain certain compounds that would not require bioactivation consequently, its observed toxicity *in vitro*.

All the extracts analyzed were cytotoxic towards the normal human liver cell (WRL 68), thus exhibiting a rather poor selectivity. This suggests some degree of toxicity when any part of this plant is ingested for the purpose of alleviating a disease condition. However, further *in vivo* studies will be required to confirm this.

All the fractions obtained from the stem of this plant were relatively less toxic against the cancer cells as compared to the crude extract. This could be attributed to the fact that the active molecules in the extracts worked in a synergistic manner (or the activity of the active compound was complemented by another compound) and individually was not that effective. On the other hand, it is possible that the solvent used for fractionation was unsuitable for the purpose. Hence the fractionation procedure did not yield any significant increase in cytotoxicity.

CONCLUSION

Data from this study suggests *C. retusa* leaf is a good source of phenolic compounds with good antioxidant properties. The stem of *C. retusa*, is effective in inhibiting the growth of Jurkat, MCF 7 and PC 3 cancer cells. However, its cytotoxicity towards normal cell (WRL 68) renders it a less desirable chemotherapeutic alternative.

Purification of *C. retusa* by Srinivas *et al.* (2014) yielded monocrotaline, which was much more toxic towards VERO (kidney) and HeLa (cervical) cancer cells. Though fractions from the stem had a lower cytotoxicity effect on cancer cells relative to the crude extract, it is possible that further purification and isolation of a pure compound from the stem of *C. retusa* is likely to improve the observed cytotoxicity on the cancer cell lines and possibly reduce its toxicity towards normal cell. This could serve as a lead to identifying and developing a potential chemotherapeutic agent against cancer.

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